Are there actually three models in the processing of pheromonal signals in mammals? The striking features of G-proteins

M. Vázquez Torres[1], P.R. Villamayor[1], E. Valderrábano[2], A. Ferreiro[2], J.M. Cifuentes[1], J.L. Rois[2], P. Sanchez-Quinteiro[1]

[1] Departamento de Anatomía, Producción Animal y Ciencias Clínicas Veterinarias
[2] Parque Zoológico Marcelle Naturaleza, Outeiro de Rei, Lugo
[1] Facultad of Veterinaria, Universidad de Santiago de Compostela, España

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Contact: paularodriguez.villamayor@usc.es

Introduction

The vomeronasal and the olfactory sensory neurons are the main specialized chemoreceptor cell types of the nasal cavity. Both types are distinct in their morphology (vomeronasal neurons possess microvilli, whereas olfactory neurons have cilia), projection areas (accessory and main olfactory bulbs, respectively for the vomeronasal system (VNS) and the main olfactory system (MOS)), and in the receptors and signal transduction components that they express.

Olfactory neurons express a family of about 1000 receptors coupled to the G-protein G_{olf}. Vomeronasal neurons do not express the classical olfactory receptors, nor do they express G_{olf}. Instead, they own two main families of specific vomeronasal receptors, V1R and V2R. V1R were identified by Dulac & Axel (1995) using a single-cell PCR strategy to find genes expressed at high levels in a subset of vomeronasal neurons. V2R was characterized few years later by Ryba & Tirindelli (1997) using a different approach, more stochastic.

Simultaneously to these mollecular findings, morphological studies in rodents and opossums VNS employing immunohistochemical labelling against the G-proteins $G_{\alpha i2}$ and $G_{\alpha o}$ determined the existence of a subtle organization of the vomeronasal receptor cells (Jia & Halpern, 1996). They actually comprised two subpopulations which followed a well defined topographic zonation, both in the VNO and the AOB. The presence of this zonation was confirmed by other markers, as some specific lectins (Shapiro et al., 1995, Salazar et al., 2001).

The presence of these G protein-specific subpopulations was soon correlated to the expression of the two main families of VRs. Thus, V1R cells express $G_{\alpha i2}$ whereas V2R cells express $G_{\alpha o}$ protein. Afterwards, G-proteins have become a useful and efficient tool to identify the presence of VR subpopulations among different species.

Altogether, these significant anatomical, microscopic and molecular differences are a reflection of the functional specialization of both systems: detection of airborne odorants by the MOS and characterization of body semiochemicals (mostly pheromones and kairomones) by the VNS. Chemical communication has evolved differently depending on the group of mammals and accordingly to their ecology, social behaviour, reproductive physiology, etc. Not suprisingly, the VRs expression has been adapted in consonance with these specific requirements. Accordingly, the widespread characterization of the G proteins in mammals has demonstrated that a wide range of species -dog, cats, goats, sheep, horses, squirrels, musk shrew, etc.- have suffered a deterioration of the V2R pathway.

At the same time, the study of the tammar wallaby *Macropus eugenii* VNS by Schneider *et al.* (2012) produced an alternative and unexpected finding. This marsupial species not only did not express both VR families, but the expressed one was the $G_{\alpha o}$, instead of $G_{\alpha i2.}$ They were not able to find $G_{\alpha i2}$ positive cells in both the VNO and AOB. They only identified $G_{\alpha o}$ positive cells in the basal VNO and in the whole length of the superficial layers of the AOB. In fact, the only image they show of anti- $G_{\alpha o}$ positive labelling in the AOB showed a very sparse labelling.

As a result of their findings they postulated the presence of a third type of vomeronasal information processing. To date, the tammar wallaby represents the only example of this striking pathway.

In order to shed light on this critical subject we have studied the structural and immunohistochemical features of the wallaby. The individuals studied by us belonged to the species *Macropus rufogriseus,* the Bennet's wallaby.

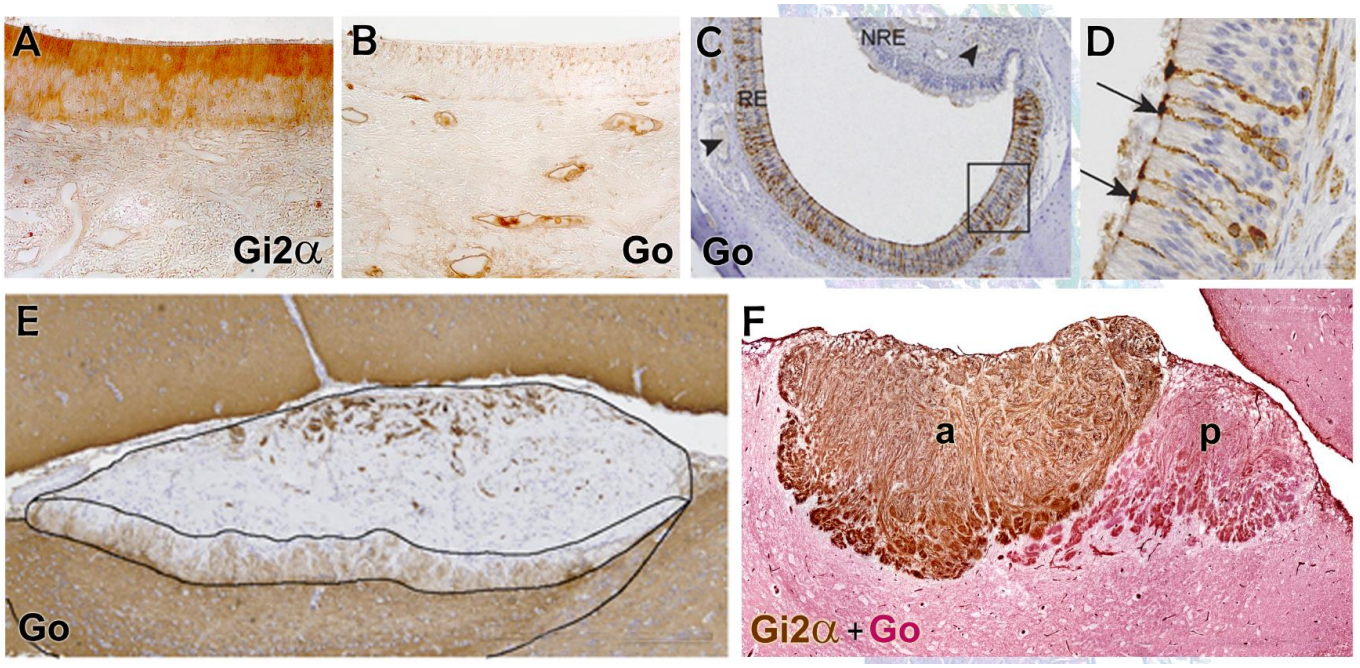


Fig. 1 Three models of G-protein expression in the VNS. (A,B) Gαi2 immunopositive expresion (A) and Gαo negative expresion (B) in the dog VNS epithelium (Salazar et al., 2013). (C,D,E) Gαo-positive cells are found in the tammar's VNS receptor epithelium (C,D) and in the AOB superficial layers (E) (Schneider et al., 2012). They were not able to find Gαi2-positive cells in both structures. (F) Segregated model: Gαi2 and Gαo co-expression in the rabbit AOB following an antero-posterior zonation (Villamayor *et al*., 2019).

Material & Methods

Three adult healthy wallabies were provided by the natural park of Marcelle Natureza (Outeiro de Rei, Lugo). All of them died from natural causes. Their heads were fixed in both neutral formalin and Bouin's flouid.

The vomeronasal organs and the olfactory bulbs (OBs) were completely dissected out to be processed. Paraffin wax embedding was the inclusion procedure chosen for all the samples. The VNOs were cut into transversal sagittal 5-8 µm sections and the OBs in sagittal sections of the same thickness. The sections were stained with

haematoxylin, Nissl and Tolivia stainings. Immunohistochemical stainings were performed in paraffin embedded sections.

Immunohistochemical protocol for paraffin-embedded tissue

All primary antibody incubations were performed at 4 °C temperature while the secondary antibodies were incubated at room temperature. Both were kept in a humid chamber during the entire procedures. Unless otherwise stated, all washing steps consisted of three successive 5 min rinses in PB.

The sections were quenched in 3% H_2O_2 for 15 min. Next, non-specific binding was blocked for 30 minutes with 2.5% horse normal serum of the Impress reagent kit Anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). The sections were then overnight incubated with the primary antibody. The next day the samples were incubated for 20 minutes with the corresponding ImmPRESS VR Polymer HRP Anti-Rabbit IgG Reagent. After rinsing in 0.2 M Tris–HCl buffer (pH 7.6) for 10 min, the sections were finally developed using DAB as chromogen and then dehydrated and mounted.

Lectin histochemical protocol for paraffin-embedded tissue

The protocol was based in the incubation with biotinylated lectins overnight and, at the second day with ABC kit from Vector. The development was done in the same way as in the IHC procedure

Results

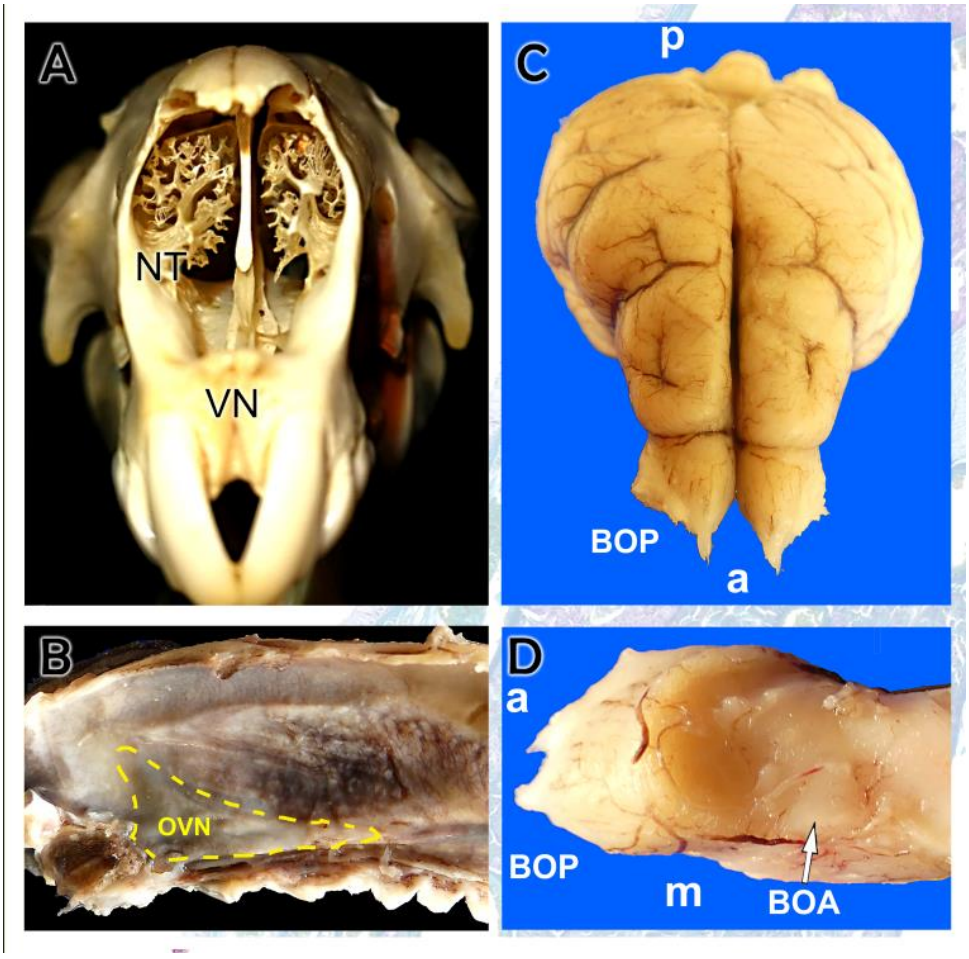


Fig. 2 Macroscopic features of the wallaby VNS.

(A) Anterior view of the nasal cavity VN: vomer bone; NT: Nasal turbinates.

(B) Lateral view of the nasal septum with the OVN projection underlined.

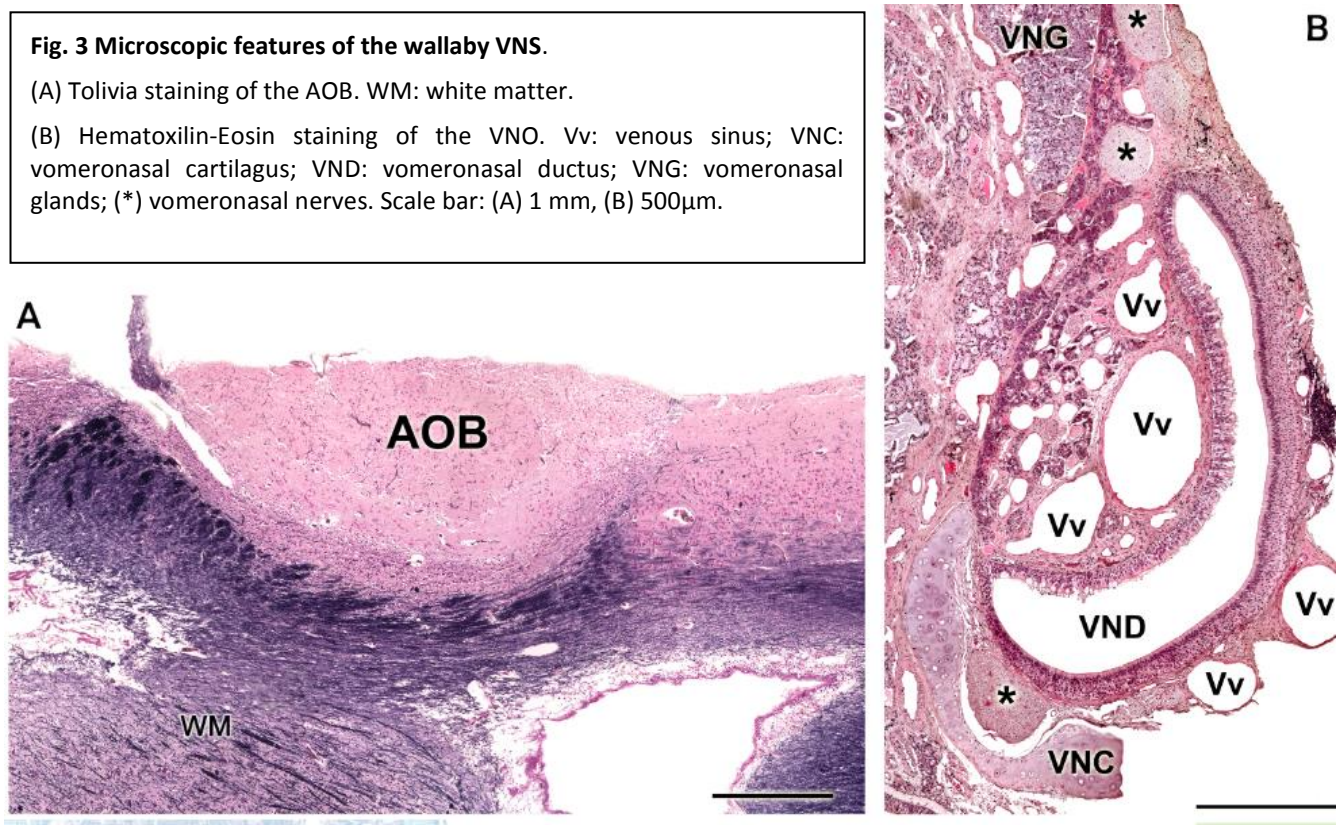
(C) Dorsal view of the brain, showing the BOP location. a: anterior; p: pos terior.

(D) Dorsomedial view of the BOP showing the location of the BOA. d: dorsal; m: medial.

Fig. 3 Microscopic features of the wallaby VNS.

(A) Tolivia staining of the AOB. WM: white matter.

(B) Hematoxilin-Eosin staining of the VNO. Vv: venous sinus; VNC: vomeronasal cartilagus; VND: vomeronasal ductus; VNG: vomeronasal glands; (*) vomeronasal nerves. Scale bar: (A) 1 mm, (B) 500µm.



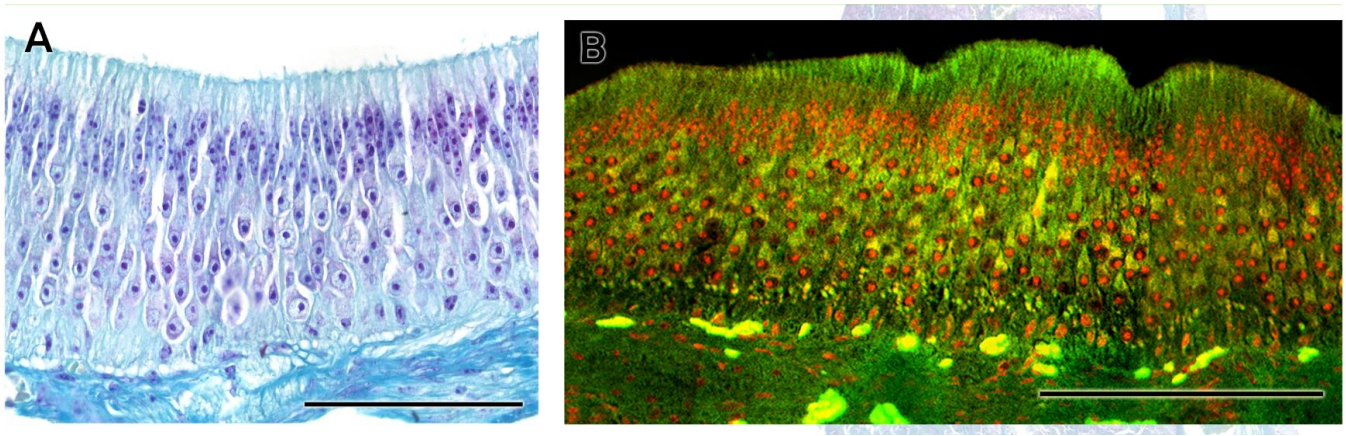


Fig. 4 Neuroepithelium of the VNO. (A) Gallego's trichome. (B) Confocal microscopy. Scale bars: 100 µm

Both the macroscopic and microscopic features of the wallaby VNS correspond to a very well-developed sensorial system. The VNO is located in the base of the nasal septum, and the AOB is easily identified macroscopically in the dorsomedial part of the MOB (Fig.2).

Histologically, the central part of the organ contains a huge vomeronasal duct. Associated to the duct there is a parenchyma very rich in glands, blood vessels and nerves (Fig.3).

The neuroepithelium of the organ is very thick and rich in neuroreceptor and sustentacular cells. Both are very easily identified using confocal imaging (Fig.4).

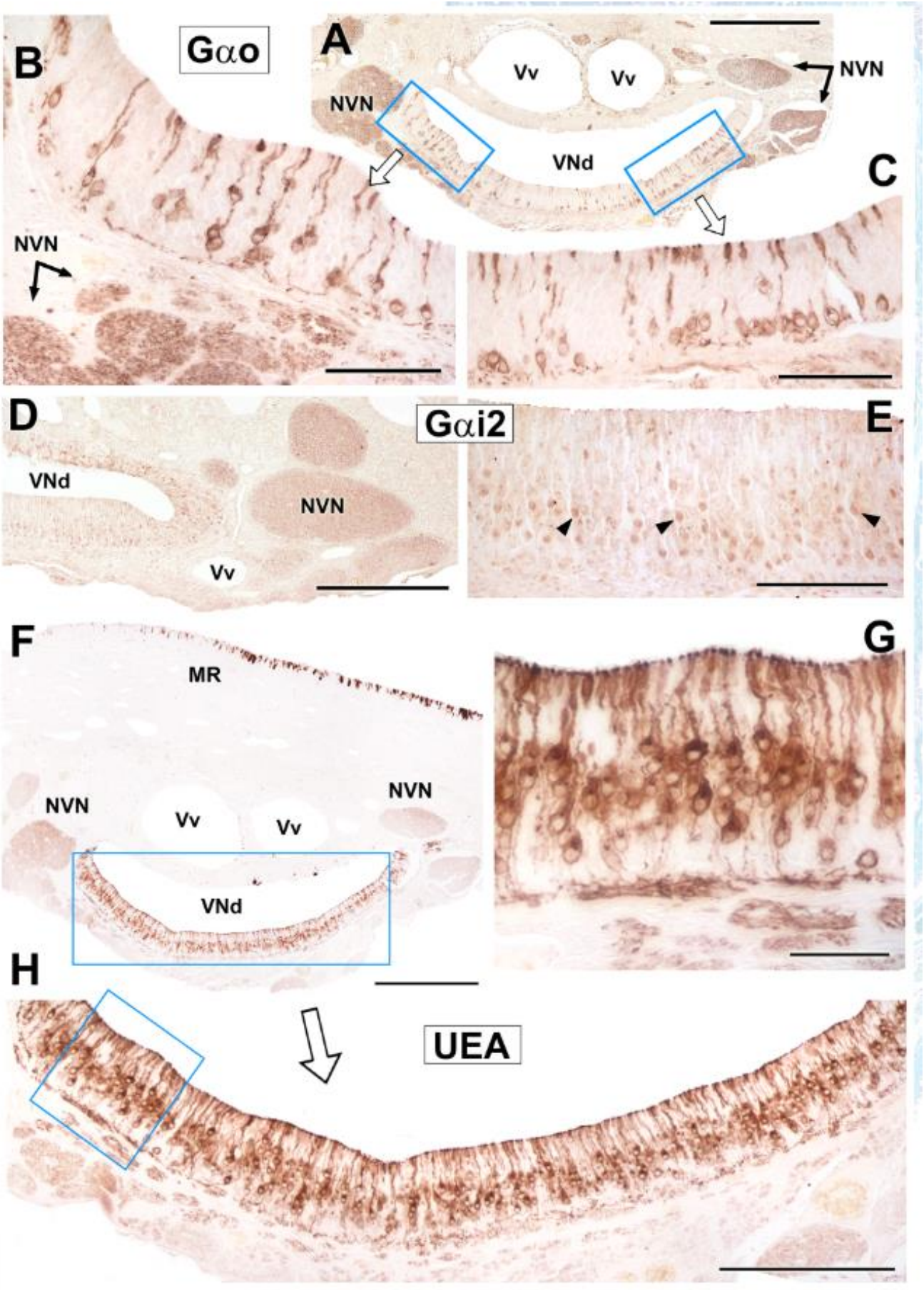


Fig. 5: IHC of the wallaby OVN. (A,B,C) Anti-Gαo: strong staining in the nerves and the entire neuroreceptor cells. (D,E) Anti-Gαi2: the staining is concentrated in the nucleus of the neuroreceptor cells. (F,G,H) UEA lectin, which stains the nerves and the neuroepithelium. In this case, the staining is concentrated in the soma and the dendrites. Scale bar: (A,D,F) 500 µm; (H) 250 µm; (B,C,E) 100 µm; (G) 50 µm.

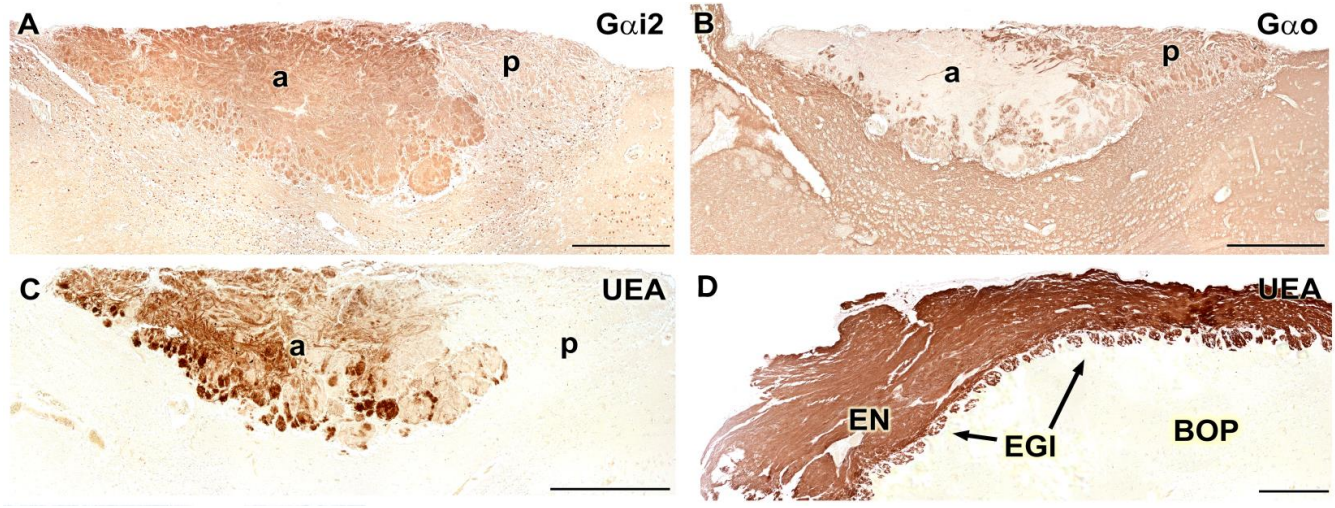


Fig. 6: IHC of the Wallaby AOB. (A) Anti-Gαi2: It defines an anterior (+) and posterior (-) zonation. (B) Anti-Gαo: In this case, it shows the complementary staining: anterior (-) and posterior (+). (C,D) UEA staining in the AOB can be compared to anti-Gαi2. Barra de escala: (A-C) 500 µm; (D) 250 µm.

The immunohistochemical labelling with antibodies against G proteins produces a strong reaction in both the VNO (Fig.5) and the AOB (Fig.6). In the neuroepithelium of the organ, anti-Gαo labels very strong and neatly a subpopulation of neuroreceptor cells, whose soma are mostly located in the basal part of the epithelium. Their long dendritic processes are labelled as well. The vomeronasal nerves in the lamina propria are also immunopositive. Their arrival to the AOB is also strongly labelled but exclusively in those nerves reaching the posterior part of the accessory bulb. The labelling comprises both the nervous and glomerular layers. The neuropil of the MOB and the deeper layers of both the anterior and posterior AOB are immunopositive.

Regarding the anti-Gαi2 the immunolabelling in the neuroepithelium is more diffuse and restricted to the soma of neuroreceptor cells. The vomeronasal nerves leaving the VNO show a strong positive reaction which is confirmed in the AOB. In this case the labelling is restricted to the superficial layers of the anterior part of the AOB. The rest of the AOB and the whole MOB are immunonegative.

The Lectin-histochemical labelling with UEA, in addition to giving a strong and diffuse labelling of the neuroepithelium, strongly stains the vomeronasal nerves and the anterior part of the AOB (Fig.6). Strikingly, this lectin does not label at all the posterior part of the AOB. The superficial layers of the MOB are strongly labelled by this marker.

Discusion

Our structural and immunohistochemical study of the VNS of the Bennet's wallaby demonstrates the presence in this species of an intact vomeronasal dual pathway, which entirely corresponds to the segregated pattern described previously in Rodents (Jia & Halpern, 1995) and Lagomorpha (Villamayor *et al.,* 2018). There is a huge presence of Gαi2 neuroreceptor cells in the vomeronasal neuroepithelium. Although the basal-apical segregation described in mice is unclear, this feature has not been described in the vomeronasal neuroepithelium of the rabbit, so it is not necessarily a feature related to the segregated model.

The antero-posterior pattern in the AOB is however very clearly outlined, following the typical Gαi2 anterior AOB, plus Gαo posterior AOB. This zonation is confirmed with the histochemical labelling with UEA which stains the anterior part of the AOB (Fig.6), but it does not label at all the posterior part of the AOB.

These results contrast with those obtained by Schneider *et al.* in the tammar wallaby and suggest that the absence of anti-Gαi2 labelling in the vomeronasal neuroepithelium, nerves and AOB, could be due to methodological issues. As the authors of this study consider, issues of specificity of the antibody against Gαi2 employed by them could explain the absence of labelling, a paradoxical result, as it is known by genomic studies that the tammar wallaby possesses V1R receptor functional genes.

In our case, we have employed an antibody widely and successfully used in numerous mammal, amphibia and fish species. Moreover, the correspondence of labelling between the pattern found in the VNO epithelium, vomeronasal nerves and AOB, and the confirmation of the zonation by means of the UEA lectin, seem to indicate the expression not only of V2R receptor, but also V1R genes in the vomeronasal pathway of the wallaby. It would be very striking that being the *Macropus eugenii* the only species described with exclusive Gαo expression, a species belonging to the same Genus as the *Macropus rufogriseus* had not experienced the same atypical deterioration of the Gαi2 pathway. For all these reasons we consider plausible to reject the existence of the proposed third model of pheromonal information processing in mamals.

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